



Published in final edited form as:

Nat Commun. ; 6: 6704. doi:10.1038/ncomms7704.

## Metabolic learning and memory formation by the brain influence systemic metabolic homeostasis

Yumin Zhang<sup>1,2,3</sup>, Gang Liu<sup>1,3</sup>, Jingqi Yan<sup>1</sup>, Yalin Zhang<sup>1</sup>, Bo Li<sup>1</sup>, and Dongsheng Cai<sup>1,\*</sup>

<sup>1</sup>Department of Molecular Pharmacology, Diabetes Research Center, Institute of Aging, Albert Einstein College of Medicine, Bronx, NY 10461, USA

<sup>2</sup>Department of Endocrinology and Metabolism, the First Hospital of Jilin University, Changchun, 130021, China

### Abstract

Metabolic homeostasis is regulated by the brain, whether this regulation involves learning and memory of metabolic information remains unexplored. Here we use a calorie-based, taste-independent learning/memory paradigm to show that *Drosophila* form metabolic memories that help balancing food choice with caloric intake; however, this metabolic learning or memory is lost under chronic high-calorie feeding. We show that loss of individual learning/memory-regulating genes causes a metabolic learning defect, leading to elevated trehalose and lipids levels. Importantly, this function of metabolic learning requires not only the mushroom body but the hypothalamus-like pars intercerebralis, while NF- $\kappa$ B activation in the pars intercerebralis mimics chronic overnutrition in that it causes metabolic learning impairment and disorders. Finally, we evaluate this concept of metabolic learning/memory in mice, suggesting the hypothalamus is involved in a form of nutritional learning and memory, which is critical for determining resistance or susceptibility to obesity. In conclusion, our data indicate the brain, and potentially the hypothalamus, direct metabolic learning and the formation of memories, which contribute to the control of systemic metabolic homeostasis.

### Introduction

Learning and memory formation are fundamental biological processes contributing to the survival of a species under changing environmental conditions. The brain is crucial for this function that is built up classically through processing the information from visual, auditory, olfactory, taste and other sensory systems. Compared with this traditional view on learning

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\*Address correspondence to: Dongsheng Cai, M.D., Ph.D., Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, Phone: 718-430-2426, Fax: 718-430-8922, [dongsheng.cai@einstein.yu.edu](mailto:dongsheng.cai@einstein.yu.edu).

<sup>3</sup>These authors equally contributed to this work.

**Author contributions:** DC conceived the hypothesis and designed the study and structures of experiments. YZ and GL co-designed and performed the experiments. JY, Ya.Z and BL provided experimental collaboration and technical assistance. DC, YZ, GL and JY did data interpretation and discussion. DC wrote the paper, and YZ and GL prepared figures and provided writing assistance.

### Competing Interests Statement

The authors declare no competing financial interests.

and memory, it is, however, unknown if the central control of metabolic homeostasis chronically involves a form of learning and memorizing post-ingestive metabolic information independent of the sensory properties of food, which we briefly refer to as “metabolic learning/memory”. Of interest, recent research showed that flies can distinguish the nutritional value from the taste of food<sup>1,2</sup>, but questions remain regarding if the brain can employ a mechanism of metabolic learning to regulate metabolic homeostasis and, if so, whether this function requires the brain region that critically mediates nutrient sensing, e.g., the hypothalamus in mammals and pars intercerebralis (PI) in *Drosophila*. Moreover, from a disease perspective, it is worth exploring if metabolic learning impairment mediates the development of metabolic diseases, especially since learning and memory disorders are often seen in overnutrition-induced diseases<sup>3</sup>.

Here, we developed a series of *Drosophila* and mouse models and obtained a body of results collectively suggesting that metabolic learning and memory formation by the brain, and potentially by the hypothalamus, is important for the central control of metabolic homeostasis, and dysfunction of this mechanism contributes to the development of metabolic diseases.

## Results

### Feeding control via metabolic learning in *Drosophila*

In this work, we developed a *Drosophila* conditioning learning and memory paradigm, based on a literature-established taste/smell-matched food recipe using the same yeast content but altered sorbitol content which provides an alternative but tasteless carbohydrate source<sup>1,2</sup>. An olfactory conditioning paradigm was used (Supplementary Fig. 1), in which volatile chemicals 3-Octanol (OCT) and 4-Methylcyclohexanol (MCH) were used as conditional stimuli, a well-established approach in the literature<sup>4</sup>, while unconditional stimulus was a normal-calorie food (NC) containing 50g/L sorbitol vs. a high-calorie food (HC) containing 200g/L sorbitol, both of which had the same yeast content (50g/L). Indeed, whether fed or fasting condition was tested, untrained *Canton-S* (CS) flies did not show any bias towards NC or HC when initially exposed to these two food types (Supplementary Fig. 2a), supporting that NC and HC had the same taste. Flies were trained to develop a learning/memory association between an odor and a food type. As elucidated in Supplementary Fig. 1, for each training cycle, flies were kept 24 hours in NC-containing vials circulated with one odor, and then 24 hours in HC-containing vials circulated with the other odor. After a designated number of training cycles, flies were tested for conditional preference between two odors in the absence of food, in which flies were loaded at the middle joint section (Supplementary Fig. 1) and their choices of OCT vs. MCH vials were recorded. This conditional preference was reflected by Preference Index, calculated as  $([\text{number of flies that traveled to vials with NC-associated odor}] - [\text{number of flies that travelled to vials with HC-associated odor}]) / [\text{total number of flies in both vials}]$ .

Results showed that following 3–6 training cycles, flies established a strong preference for NC-associated odor but a strong discrimination against HC-associated odor (Fig. 1a&b). Also, we alternated these two odors in association with NC vs. HC, and confirmed that flies always developed a preference to NC-associated odor whether MCH or OCT was matched

(Supplementary Fig. 2b). We also confirmed that Preference Index was not affected whether the flies were initially placed to NC or HC (Supplementary Fig. 2c). In addition, we considered that the training course was associated with age increase, and thus tested if this age increase could have an impact on odor preference. To do so, we compared untrained flies at the ages matched with the flies prior to vs. post training, and verified that this age increase per se did not lead to a bias for an odor (Supplementary Fig. 2d). Finally, to additionally evaluate this finding, we compared NC with a range of food sorbitol contents, including a low concentration (20g/L) and an extreme high concentration (300g/L) while the yeast content was the same (50g/L). We found that flies consistently preferred for NC-associated odor while discriminated against high-calorie or low-calorie food (Fig. 1a). Using food intake as a readout measurement, we observed that while untrained flies ate NC and HC similarly at cycle 0, they volunteered to eat a less amount of HC than NC after 3–6 training cycles (Fig. 1c). Taken together, these findings indicate that flies employ metabolic learning to form metabolic memory and thus decrease the exposure to overnutritional environment when normal nutritional environment is available.

### Impaired metabolic learning/memory by chronic overnutrition

As learning and memory disorders are often displayed in chronic overnutrition-induced diseases<sup>3</sup>, we were curious if this relationship might apply to metabolic learning and memory formation. To test this idea, we subjected flies to a prolonged training course (up to 21 training cycles) and thus introduced a long-duration although intermittent HC feeding in this behavioral test. As shown in Fig. 1b, flies decreased their preference to NC after 12 training cycles which involved 12-day exposure to HC, and barely distinguished NC vs. HC following 15–18 training cycles which involved a longer period of HC exposure. Following this finding, we asked if the age increase during this training might contribute to metabolic learning impairment. To answer this question, we tested flies at an age which was matched with 18 training cycles shown in Fig. 1b, but they received only 6 training cycles prior to Preference Index measurement. As shown in Supplementary Fig. 3, these flies still showed a strong level of metabolic learning after 6 training cycles, indicating that the age increase during the training did not have an influence on learning and memory. We also measured food intake, showing that while flies ate a less amount of HC than NC during 3–9 training cycles, this difference between two diets disappeared after 12–15 training cycles (Fig. 1c). Altogether, chronic exposure to overnutritional environment has an effect in impairing metabolic learning, pretty much like the scenario in which chronic overnutrition leads to learning and memory disorders as appreciated in the literature<sup>3</sup>.

Next, since impaired metabolic learning was associated with increased high-caloric intake (Fig. 1c), we wanted to determine if this increase in dietary caloric intake on a chronic basis could have a negative effect on metabolic physiology. To do so, we measured trehalose, a glucose disaccharide synthesized from intracellular glucose in the fat body, which is then secreted into the hemolymph. Indeed, increases of sorbitol content in the food led to increased trehalose levels in the body in a dose-dependent manner (Fig. 1d), and these increases in the body were consistent with increased trehalose levels in the hemolymph (Supplementary Fig. 4). Also consistently, increases of sorbitol content in the food led to a dose-dependent increase in triglycerides (TG) levels in the body (Fig. 1e). Hence, these flies

developed hyperglycemia and hyperlipidemia which collectively resemble diabetic or pre-diabetic changes in mammals under chronic overnutrition. Moreover, as shown in Fig. 1f, we consistently found that chronic treatment of high-sorbitol food led to downregulation of many learning/memory-regulating genes in the brain, including *rutabaga* (*rut*), *dunce* (*dnc*), *tequila*, *sarah* (*sra*), *syndrome* (*syn*) and *amnesiac* (*amn*), all of which are known important for learning and memory formation in *Drosophila*<sup>5–8</sup>. Finally, based on the manifestation of diabetic and lipid disorders in these flies, the general health of these animals should be predicted to decline substantially; indeed, when maintained on this high-sorbitol food, these flies displayed lifespan shortening (Fig. 1g) and poor fecundity (Fig. 1h). This long-term compromise in health was at least an outcome of joint effects from metabolic learning defect and other independent mechanisms induced by chronic overnutrition. In summary, impaired metabolic learning and memory formation under chronic overnutrition have a negative impact on physiology and health.

### Requirement of *rut* gene in metabolic learning/memory

Learning and memory formation has short-term and long-term forms, and according to the literature<sup>8–10</sup>, short-term learning and memory in the brain is importantly mediated by *rut* gene, which encodes a type I adenylyl cyclase that functions to associate unconditional with conditional stimuli. It has been documented that *rut* is expressed only in a few brain regions, in particular, the mushroom body (MB)<sup>11</sup>. Using *in situ* hybridization, we confirmed that *rut* was expressed predominantly in the MB, while its expression in the PI was also appreciable (Fig. 2a). Supported by this background information, we decided to explore if a mutation of this gene could affect metabolic learning. To do so, we studied if metabolic learning could be affected by a *rut* mutation in a *Drosophila* mutant strain *rut*<sup>2080</sup> which was reported to have impaired short-term learning<sup>12–14</sup>. Technically, *rut*<sup>2080</sup> flies have normal visual<sup>14</sup>, olfactory<sup>15</sup>, and gustatory<sup>16</sup> functions, and were therefore suitable for the conditional training paradigm in our study. We verified that *rut*<sup>2080</sup> mutant had normal climbing activities, reproductive function and egg viability when maintained on a regular food (Supplementary Fig. 5), again supporting that these animals are appropriate for our behavioral test. Under normal food conditions without training, *rut*<sup>2080</sup> mutants and *wild-type* (*wt*) controls showed similar food intake and body weight during a 3-week follow-up. Also, when responding to normal- vs. high-sorbitol food, they did not show an immediate preference to a food type (Supplementary Fig. 6). Subsequently, these mutant and *wt* animals were subjected to the training demonstrated in Supplementary Fig. 1. Data show that *wt* controls showed metabolic learning and memory formation at training cycle 7, but *rut*<sup>2080</sup> mutants failed to do so (Fig. 2b–d). The failure of developing metabolic learning in these mutants was also indicated according to food intake measurement, showing that unlike *wt* which discriminated against high-sorbitol food following 6 training cycles (Supplementary Fig. 7a), *rut*<sup>2080</sup> did not have this response despite the same training (Supplementary Fig. 7b). Accordingly, compared to *wt* controls, *rut*<sup>2080</sup> showed significant increases in trehalose and TG contents over this training course (Fig. 2e&f). Altogether, these data suggest that the *rut* gene is required in the formation of metabolic learning and memory formation.

To further address if the neural system is important for the phenotype that we observed in *rut*<sup>2080</sup>, we restored the wild-type copy of *rut* gene selectively in the neural system of *rut*<sup>2080</sup>. This restoration was achieved by crossing *rut*<sup>2080</sup>; *UAS-rut*<sup>+</sup> flies<sup>12–14,17</sup> to flies that expressed *GAL4* under the control of a pan-neural *elav* promoter. Progenies, which had neuronal *rut*<sup>+</sup> expression due to *GAL4*-induced UAS activation, were collected and analyzed to determine if a rescue effect could be achieved. Data revealed that restoration of *rut* in the neural system led to a significant reversal of metabolic learning defect in *rut*<sup>2080</sup> mutants (Fig. 2g–i). Moreover, this neural restoration of *rut* significantly protected against trehalose and TG increases in *rut*<sup>2080</sup> mutants (Supplementary Fig. 8). Hence, these data lend a support to the model that *rut* in the brain is important for metabolic learning and memory formation.

### Requirement of *dnc* and *tequila* in metabolic learning/memory

In addition to *rut*, we studied several other learning/memory-regulating genes, including *dnc* and *tequila* which are also important for learning and memory formation<sup>6,7,18–22</sup>. Regarding *dnc*, it encodes a phosphodiesterase enzyme that hydrolyzes cAMP, and coordinates with the learning/memory-regulatory function of *rut* to maintain cAMP in a normal range for short-term memory<sup>7,18,19</sup>. In our study, we tested *dunce*<sup>1</sup> flies, a line of *dnc* mutant *Drosophila* established in the literature<sup>20,21</sup>. When maintained on a regular food, we confirmed that *dunce*<sup>1</sup> had normal locomotion (Supplementary Fig. 5a), although female *dunce*<sup>1</sup> had lower reproductive activities (Supplementary Fig. 5b&c). Under normal conditions, untrained *dunce*<sup>1</sup> and *wt* flies showed similar food intake and body weight, and no immediate preference for food types was confirmed when exposed to normal- vs. high-sorbitol food (Supplementary Fig. 6). However, following training cycles, we found that just like *rut*<sup>2080</sup>, these mutant flies showed a loss of conditional preference for normal-sorbitol food compared to *wt* flies (Fig. 3a–c). Also, unlike *wt* which developed a discrimination against high-sorbitol food over 6 training cycles (Supplementary Fig. 7a), *dunce*<sup>1</sup> did not have this discrimination regardless of the same training (Supplementary Fig. 7c), and this effect was associated with increased trehalose and TG levels (Fig. 3d&e). In parallel with *dnc*, *tequila* gene was studied as well, which encodes a neurotrypsin ortholog; based on a *tequila* mutant line (*teq*<sup>-</sup>), this gene is known to be important for long-term memory<sup>6,22</sup>. After having confirmed the suitability of this fly model for our behavioral test (Supplementary Fig. 5& Supplementary Fig. 6), we subjected *teq*<sup>-</sup> and *wt* to the learning and memory training, and revealed that these mutants also showed a significant defect in metabolic learning (Fig. 4a–c), which was accompanied by metabolic disorders (Fig. 4d&e). In summary, multiple learning/memory-regulating genes are involved in metabolic learning and memory formation.

### Requirement of the MB in metabolic learning/memory

To understand the neuronal types which potentially convey metabolic information to a learning process, we first investigated neurons in the MB, a brain region which is classically known for inducing learning and memory in *Drosophila*<sup>23</sup>. As established, *rut* and *dnc* are two key genes that regulate cAMP balance in the MB, and disruption of each in the MB is sufficient to impair learning and memory formation<sup>24</sup>. Thus, to examine if the MB neurons are required for metabolic learning, we employed an MB-specific *GAL4* line (*17D-GAL4*) to

inhibit *rut* or *dnc* gene by crossing this *GAL4* strain with either *UAS-rut<sup>RNAi</sup>* or *UAS-dnc<sup>RNAi</sup>* flies to create *17D-GAL4/UAS-rut<sup>RNAi</sup>* and *17D-GAL4/UAS-dnc<sup>RNAi</sup>* flies, respectively. As described in Methods, these *UAS-RNAi* strains were created using the VALIUM10 vector system, which is known to sufficiently induce RNAi knockdown without additionally introducing *dicer*<sup>25</sup>. Our subsequent study revealed that both of these fly lines showed the absence of metabolic learning under training (Fig. 5a&b). In addition to these two fly models, we generated a third one in which we directly targeted PKA, a key component downstream of the protein encoded by *rut*, which is needed for the function of MB in inducing learning and memory<sup>26</sup>. To do so, we used inhibitory PKA (PKA<sup>inh</sup>), which has a mutant cAMP-binding site and thus inhibits the function of *rut* in regulating learning and memory formation<sup>27</sup>. We therefore generated an MB-specific transgenic line with PKA<sup>inh</sup> expression by crossing *17D-GAL4* with *UAS-PKA<sup>inh</sup>* flies, resulting in *17D-GAL4/UAS-PKA<sup>inh</sup>* flies. By examining these mutants, we found that PKA inhibition in this fly model led to a partial reduction in metabolic learning and memory formation (Fig. 5c). Finally, we profiled the metabolic physiology of these three fly models vs. their controls, and results consistently demonstrated that the trehalose and TG levels in all these mutant animals significantly increased, although to different degrees (Supplementary Fig 9a&b). In summary, the MB is involved in mediating metabolic learning and memory formation in *Drosophila*.

### Requirement of the PI region in metabolic learning/memory

Equivalent to mammalian hypothalamic neurons, *Drosophila* has a group of endocrine neurons in the hypothalamus-like PI region, which produce *Drosophila* insulin-like peptides (dilps) and have been genetically targeted in *dilp2-GAL4* flies which provide a *Drosophila* tool to study this neuronal population<sup>28–31</sup>. Indeed, defects in these *dilp2-GAL4*-defined neurons can cause many metabolic disorders including diabetes and related diseases<sup>30,31</sup>. This knowledge led us to hypothesize that these *dilp2-GAL4*-defined PI neurons might participate in metabolic learning. To test this hypothesis, we inhibited *rut* in these neurons by crossing *dilp2-GAL4* with *UAS-rut<sup>RNAi</sup>* flies to create *dilp2-GAL4/UAS-rut<sup>RNAi</sup>* flies. Using our training paradigm, we observed that these mutant flies showed poor function of metabolic learning (Fig. 5d). As a result of this defect, these mutant flies showed significant increases in trehalose and TG levels (Fig. 5e&f). To further evaluate the role of these neurons in metabolic learning and memory formation, we independently generated another fly model in which PKA<sup>inh</sup> was expressed specifically in *dilp2-GAL4*-defined neurons. To do so, we crossed *dilp2-GAL4* with *UAS-PKA<sup>inh</sup>* to generate *dilp2-GAL4/UAS-PKA<sup>inh</sup>* flies. These mutant flies also demonstrated a defective function of metabolic learning (Fig. 5g) and increased levels in trehalose and TG (Fig. 5h&i) during the training course. These results suggest that *dilp2*-expressing neurons in the PI region are necessary for the process of metabolic learning. Admittedly, this study did not address if the role of PI neurons in regulating metabolic learning is independent of the signaling of *dilp2* and perhaps other dilps. Regardless, this possibility can be reasoned, considering that PKA was reported to negatively affect insulin/IGF signaling in flies<sup>32</sup>, and our results suggest that PKA in the *dilp2-GAL4*-defined neurons is needed for metabolic learning. Thus, future studies are needed to study if dilps might participate in the function of PI in mediating metabolic

learning. In summary, certain neurons in the PI work cooperatively with the MB to mediate metabolic learning and memory formation.

### Overnutrition impairs metabolic learning via inflammation

Recently using mouse models, we have established that chronic overnutrition induces NF- $\kappa$ B-dependent hypothalamic inflammation and causes obesity and diabetes<sup>33,34</sup>. In relation with this background, our studies in this work elucidated that metabolic learning not only requires the hypothalamus-like PI region but also can be impaired under chronic overnutrition. Thus, we logically asked if the NF- $\kappa$ B-induced inflammatory pathway might link overnutrition to metabolic learning impairment in *Drosophila*. Regarding the NF- $\kappa$ B pathway, three NF- $\kappa$ B transcription factors have been identified in *Drosophila*, including *dorsal* (*dl*), *disordered facets* (*dif*) and *Relish* (*Rel*), which represent the key regulators of immune response<sup>35</sup>. Results showed that while chronic high-calorie feeding decreased the expression of leaning/memory genes in flies (Fig. 1f), it increased the brain expression levels of NF- $\kappa$ B pathway components including *dl*, *dif* and *Rel* as well as many proinflammatory cytokine genes such as *outstretched* (*os*), *unpaired 2* (*upd2*), and *eiger* (*egr*) (Fig. 6a). Although we do not predict that these increases are responsible for NF- $\kappa$ B activation, these changes clearly indicate an inflammatory state which can reflect NF- $\kappa$ B activation. These effects of high sorbitol feeding were specific to the brain, since the expression levels of these genes in the periphery of the body did not show significant changes (Supplementary Fig. 10). Hence, these findings triggered us to question if metabolic learning could be affected by NF- $\kappa$ B in the brain and, in particular, the PI in the brain. To answer this question, we generated a *Drosophila* model with neural NF- $\kappa$ B activation, by crossing *elav-GAL4* with *UAS-dl* flies. Supportedly, data revealed that *elav-GAL4/UAS-dl* flies showed an impairment in metabolic learning and memory formation (Fig. 6b), and this defect was consistently associated with trehalose and lipid disorders (Supplementary Fig. 11).

Next, we narrowed down to *dilp2-GAL4*-defined neurons in the PI, and thus generated two independent lines of NF- $\kappa$ B activation specifically in these neurons, through crossing *dilp2-GAL4* flies with either *UAS-dl* or *UAS-Rel* flies, resulting in *dilp2-GAL4/UAS-dl* and *dilp2-GAL4/UAS-Rel* flies, respectively. These flies and their matched controls were examined using the training paradigm described above, and we found that both of these mutant lines showed impaired metabolic learning as well as increases in trehalose and TG levels (Fig. 6c–h), confirming that NF- $\kappa$ B in the PI region is a link between chronic overnutrition and its effect in impairing metabolic learning. Finally, we studied if NF- $\kappa$ B inhibition in these PI neurons could offer a protective effect for improving metabolic learning and memory formation under chronic overnutrition. To do so, we created a fly model with *dilp2* neuron-specific overexpression of *cactus* (*cact*), since Cactus is homologous to vertebrate I $\kappa$ B $\alpha$  protein which is a specific and potent inhibitor of NF- $\kappa$ B signaling<sup>36–38</sup>. This genetic model was created through crossing *dilp2-GAL4* with *UAS-cact* flies and was subjected to a long-duration exposure to high-sorbitol food using a 4-week training course. As similarly observed in Fig. 1b, control flies showed a significant defect in metabolic learning and memory formation after 14 training cycles; in contrast, *dilp2-GAL4/UAS-cact* flies still maintained a good level of metabolic learning (Fig. 6i). In alignment with this protective

effect, these mutant flies exhibited counteraction against overnutrition-induced trehalose and TG increases during this long training course (Fig. 6j&k). Taken together, activation of NF- $\kappa$ B pathway in the brain and PI region in particular, has a negative impact on metabolic learning and memory formation.

### Hypothalamic learning/memory genes in mice with obesity

With great interest, we employed a mouse model to evaluate the potential involvement of the hypothalamus in metabolic learning. To do so, we designed a study in which *A/J* mice and *C57BL/6J* mice were compared, because these two strains are resistant against and prone to dietary obesity, respectively<sup>39</sup>, and they have different nutritional preferences when subjected to free choice between high-fat and low-fat diets<sup>40,41</sup>. Specifically, we analyzed these mice, in order to explore if the different metabolic responses between these two strains might be related to different brain programs of learning and memory. In our experiment, our examination was focused on a list of learning/memory-regulating genes, including *adenylate cyclase 8 (Adcy8)*, *SH3/ankyrin domain gene 3 (Shank3)*, *synaptosomal-associated protein 25 (Snap25)*, *discs large homolog 3 and 4 (Dlg3, 4)*, *calcium/calmodulin-dependent protein kinase II  $\alpha$ ,  $\beta$  and  $\gamma$  (Camk2a, b, g)*, and *activity regulated cytoskeletal-associated protein (Arc)*, each of which is important for learning and memory formation<sup>42–48</sup>. Indeed, we confirmed that these genes were highly expressed in the hippocampus of mice, but surprisingly, the hippocampal expression levels of these genes did not much differ between *A/J* and *C57BL/6J* mice (Fig. 7a). When the hypothalamus was analyzed, a number of these genes were found to strongly express in the hypothalamus and, importantly, these genes were differentially expressed in the hypothalamus of *A/J* vs. *C57BL/6J* mice. As shown in Fig. 7b, we found that *Adcy8*, *Snap25* and *Camk2g* were downregulated while *Shank3*, *Dlg4* and *Arc* were upregulated in the hypothalamus of *C57BL/6J* mice compared to *A/J* mice. Supported by this finding, we asked if some of these changes could be induced in a separate mouse model which suffers from obesity development. We therefore employed chronic high-fat diet (HFD) feeding, since it is an important etiological factor for dietary obesity and related metabolic disorders. Results showed that compared to normal chow feeding, 3-month HFD feeding barely affected the hippocampal expression of these genes except *Dlg4* (Fig. 7c), but broadly affected the expression of these genes including *Adcy8*, *Shank3*, *Snap25*, *Dlg3*, *Dlg4*, *Camk2b* and *Arc* in the hypothalamus (Fig. 7d). By analogy, these HFD-induced hypothalamic changes significantly reflected the difference in the hypothalamus of *A/J* vs. *C57BL/6J* mice. These molecular evidences provided a suggestion to the concept that the hypothalamus is involved in a form of learning and memory formation in regulating body weight control and affecting obesity development.

### Nutritional learning/memory in mice with obesity resistance

Following the molecular study presented above, we switched to physiological study in which a behavioral paradigm was used to test if *A/J* and *C57BL/6J* mice have different learning and memory to distinguish normal vs. high-calorie food. First, these mice were examined via a visual cliff test, showing that *A/J* and *C57BL/6J* mice had the same safe responses (*A/J*: 84.37 $\pm$ 2.61% vs. *C57BL/6J*: 83.13 $\pm$ 3.43%). This result indicated that these two groups of animals had required vision for performing our behavioral test. Subsequently, using a modified method according to the literature<sup>49–52</sup>, we associated a green vs. blue



symbol with normal diet (ND, 10% kcal fat) vs. HFD (45% kcal fat), two diets having the same color (red), the same sucrose content and matched nutrient composition except for the fat to carbohydrate ratio. Mice were subjected to a training of learning and memory formation followed by measuring the preference index, as detailed in Methods. Data show that at the beginning of this training, *A/J* and *C57BL/6J* mice did not have an evident bias towards HFD or ND (Fig. 8a&b). However, by training cycle 6, *A/J* mice showed memory formation in favor of choosing ND, with this effect being maintained during training cycles 6–12 (Fig. 8a). Thus, despite HFD having a strong rewarding property<sup>53</sup>, this reward seemed to be overridden by a form of memory in *A/J* mice, although it was unclear if ND-induced appetitive memory or HFD-induced aversive memory was accountable. On the other hand, we noted that over a long training course (15 cycles), this learning and memory function in *A/J* mice declined (Fig. 8a). We speculate that prolonged exposure to HFD led to an impairment in this form of memory, which weakened its counterbalance against HFD-induced reward. In contrast, throughout the training, *C57BL/6J* mice failed to establish this memory of favoring ND preference (Fig. 8b), implicating that this mouse strain has defects in generating this form of memory. In the meanwhile, HFD-induced reward response was clearly induced in *C57BL/6J* mice, as these animals showed a preference for HFD following 3–6 training cycles. Altogether, these results suggested that there might be a form of memory in mammals contributing to metabolic control in a manner which differs from the memory mechanism in HFD-promoted rewarding response.

To explore if this particular memory contributes to the control of metabolic physiology, we applied the same learning and memory training to *A/J* vs. *C57BL/6J* mice and longitudinally profiled food intake and body weight gain in these mice. To control this experiment, we confirmed that these mice had comparable food intake under chow feeding prior to training (*A/J*: 3.40±0.48g vs. *C57BL/6J*: 3.71±0.26g). Following 3 training cycles, *A/J* mice exhibited a gradual increase in ND intake (Fig. 8c) while a gradual decrease in HFD intake (Fig. 8d), resulting in increases in the ratio of ND to HFD intake. In contrast, *C57BL/6J* mice showed a gradual decrease in ND intake and an increase in HFD intake after 3 training cycles (Fig. 8c&d). Further, *C57BL/6J* mice displayed a greater degree of body weight gain compared to *A/J* mice following the completion of 12 training cycles (Fig. 8e). As a result of obesity resistance in *A/J* mice vs. obesity susceptibility in *C57BL/6J* mice, these two strains showed phenotypic differences in light of obesity-associated metabolic disorders such as hyperlipidemia and glucose intolerance. Before training, these two strains had comparable levels of circulating free fatty acids (Fig. 8f) and comparable glucose tolerance (Fig. 8g). However, after 6 weeks of training, *C57BL/6J* mice showed increases in circulating free fatty acids (Fig. 8f) and glucose intolerance (Fig. 8g), which was clearly a result of HFD exposure during the training course. In contrast, *A/J* mice were protected from developing these metabolic disorders (Fig. 8f&g). In summary, these findings suggested that the central control of metabolic homeostasis probably involves a form of nutritional memory which is dissociable from HFD reward-induced feeding response, but the mechanism of forming this memory sensitively succumbs to the disruption under chronic overnutrition especially HFD feeding.

## Discussion

In this work, we performed a series of studies in *Drosophila* and discovered that these animals temporarily develop metabolic learning to balance food choice with caloric intake. In *Drosophila* research, sugar has often been used for studying the appetitive reward value of food taste. Of interest, recent research has suggested that fruit flies can distinguish caloric values from the taste property of food<sup>1,2</sup>. Here, using tasteless sorbitol as a carbohydrate source to generate an environmental condition which contained normal-calorie vs. high-calorie food, we revealed that *Drosophila* can develop a form of metabolic learning and memory independently of taste, by which flies are guided to have a preference for normal-caloric environment rather than high-caloric environment. However, this form of metabolic memory does not seem robust, as it is vulnerably diminished under genetic or environmental influences. We postulate that this vulnerability to overnutrition is particularly prominent for mammals (such as *C57BL/6J* mice), and overnutritional reward-induced excess in caloric intake can quickly become dominant. This effect can be consistently induced in *Drosophila* when learning/memory-regulating genes are inhibited in the brain or the hypothalamus-like PI region. We observed that each of these genetic disruptions led to impaired metabolic learning, resulting in increased caloric intake and, on a chronic basis, the development of lipid excess and diabetes-like phenotype. Indeed, it has been documented that chronic high-sugar feeding is sufficient to cause insulin resistance, obesity and diabetes in *Drosophila*<sup>54,55</sup>. It is yet unclear if this metabolic learning can induce an appetitive memory of normal-caloric environment or an aversive memory of high-caloric environment. Regardless, our findings in this work has provoked us to raise a stimulating question, that is, if this form of metabolic learning and memory is present in the mammals and if so, if this mechanism can be consolidated to improve the control of metabolic physiology and prevent against diseases. Our mouse studies in this work may provide an initial support to this concept and strategy, but clearly, in-depth future research is much needed.

In light of the underlying neural basis for this metabolic learning, our study indicates that multiple brain regions are required, including the hypothalamus-like PI region in addition to the MB (equivalent to the hippocampus in mammals) which is classically needed for learning and memory formation. Anatomically, the PI region is located in the unpaired antero-medial domain of the protocerebral cortex, which is near the calyces of the MB and the dorsal part of central complex (another brain region for regulating learning and memory)<sup>56</sup>. Functionally, the PI region has been demonstrated to coordinate with the MB in regulating various physiological activities in *Drosophila*<sup>57-59</sup>. Thus, it is very possible that some PI neurons present nutritional information to the MB and thus induce metabolic learning and memory formation. However, the underlying detailed mechanism is still unknown, especially if this process involves a role of dilps, which represent the prototypical neuropeptides produced by the PI neurons. Considering that the PI region in flies is equivalent to mammalian hypothalamus<sup>60-62</sup>, we extended this study to mouse models by comparatively analyzing *A/J* vs. *C57BL/6J* mice – which are known to have different diet preference as well as different susceptibilities to obesity development<sup>63</sup>. We revealed that while *A/J* mice showed a learning process of distinguishing normal-calorie vs. high-calorie food, *C57BL/6J* mice failed to do so. It is particularly notable that this difference of learning

and memory between these two strains is associated with differential expression profiles of learning/memory genes in the hypothalamus rather than the hippocampus. This finding, in conjunction with our *Drosophila* study, highlights a potential that the hypothalamus has a unique role in mediating metabolic learning and memory formation. Although the mouse experiments cannot exclude the impacts from the taste/smell properties of the studied food, the results demonstrated that there is a form of nutritional memory which seems dissociable from the memory of overnutritional reward. These initial observations in mice lend an agreement with our findings in *Drosophila*, suggesting that the brain and potentially the hypothalamus can link nutritional environment to a form of metabolic learning and memory.

From a disease perspective, metabolic learning in *Drosophila* is impaired under chronic overnutrition, and our mouse study was in line with this understanding. This response to overnutrition is useful when famine is outstanding, but it is a dilemma when metabolic disease is of concern, much like the scenario pertaining to leptin resistance under chronic overnutrition whereas an increase in leptin sensitivity is demanded to reduce obesity<sup>33</sup>. Recently, we have established that NF- $\kappa$ B-dependent hypothalamic inflammation links chronic overnutrition to the central dysregulation of metabolic balance<sup>33</sup>. Here in this study, we showed that activation of NF- $\kappa$ B pathway in the PI region weakened the function of metabolic learning and, conversely, NF- $\kappa$ B inhibition in this region provided a protective effect against chronic overnutrition-impaired metabolic learning. These findings are in alignment with the literature, for example, pan-neuronal NF- $\kappa$ B inhibition was shown to improve activity-dependent synaptic signaling and cognitive function including learning and memory formation<sup>64</sup>, and persistent NF- $\kappa$ B activation inhibits neuronal survival and the function of learning and memory formation<sup>65</sup>. Hence, overnutrition-induced neural NF- $\kappa$ B activation has a negative impact on metabolic learning and memory formation in regulating metabolic homeostasis.

To summarize the findings in this work, we performed a series of behavioral studies and revealed that *Drosophila* have a form of metabolic learning and memory through which the flies are directed to balance food choice with caloric intake in relevant environments. Several learning/memory-regulating genes including *rut*, *dnc*, and *tequila* are involved in this function, and brain regions include the PI in addition to the MB are required to induce this mechanism. On the other hand, metabolic learning is impaired under chronic overnutrition through NF- $\kappa$ B activation, leading to excess exposure to calorie-enriched environment which causes metabolic disorders. Overall, as elucidated in Fig. 9, metabolic learning and memory formation by the brain and potentially the hypothalamus plays a role in controlling metabolic homeostasis.

## Materials and Methods

### Fly strains and culture

The following transgenic and mutant flies were used: *dnc*<sup>1</sup>, *rut*<sup>2080</sup> and *teq*<sup>f01792</sup> (gifts from S. Waddell, University of Massachusetts Medical School), *UAS-cact* (gift from S. Govind-City University of New York), *rut*<sup>2080</sup>; *UAS-rut*<sup>+</sup> (#9405), *elav-GAL4/cyo* (#8765), *dilp2-GAL4/cyo* (#37516), *17d-GAL4* (#51631), *UAS-PKA*<sup>inh</sup> (#35550), *UAS-rut*<sup>RNAi</sup> (#27035), *UAS-dnc*<sup>RNAi</sup> (#27250), *UAS-Rel* (#9459), *UAS-dl* (#9319) were obtained from the

Bloomington Drosophila Stock Centre (Indiana University, Bloomington, IN, USA). *Canton-S* flies were used as *wild-type* controls to *dnc*<sup>1</sup>, *rut*<sup>2080</sup> and *teq*<sup>#01792</sup>. Heterozygous controls were obtained by crossing *GAL4* driver or *UAS* effector to *w1118*. All RNAi strains were generated using the VALIUM10 system, a genetic approach which has been established to induce RNAi knockdown without requiring additional introduction of *dicer*<sup>25</sup>. Flies were maintained on standard cornmeal food at 25°C and 60% relative humidity under a 12:12 h light/dark cycle, and standard food containing 50 g/L yeast and a designed content of sorbitol (Sigma) which replaced sucrose were used for the indicated experiments.

### Fly behaviors and phenotyping

Fly olfactory conditioning test was performed according to a method adapted from the literature<sup>66</sup>. Briefly, a training tube contained a fly diet was supplied with an odor (OCT or MCH) as elucidated in Supplementary Fig. 1, and odor air flow was kept constantly at the speed of 15 ml per second. For each training cycle, 50~100 flies were placed in a training tube with a specific food–odor combination for 24 hours, and switched to a second training tube with a different food–odor combination for 24 hours. The initial placement of the flies in NC or HC food was randomized. After the designed training cycles were completed, flies were subjected to preference test: following a 30-min rest in an odor-free tube, they were loaded into the odor-free middle zone of test apparatus through which flies freely chose to move into an odor-containing tube on each side. Flies were given 15 minutes to choose their preferred odor. The Preference Index value in each test was calculated as the number of flies that chose normal-calorie food-associated odor subtracted by the number of flies that chose high or low-calorie food-associated odor divided by the sum of these numbers. Food intake of flies was determined using a previously established method<sup>67</sup>. Briefly, 10 flies per sample were fed with 2.5% blue dye-containing food for 30 min, and transferred to new tubes and homogenized in 1 ml water and filtered with 0.22 µm Millex filter. The liquid phase of homogenized samples was measured for absorbance at OD 629 nm with spectrophotometer. Flies from the same groups without dye food were used as baseline control. Body weight of flies was measured using 10 flies per sample via an analytical balancer SI-114 (Denver Instrument). To determine fecundity, eggs were collected and counted from a vial containing 5–10 mated females over 24-hour period at day 6, and following hatching, subsequent hatched larvae were counted. These flies were fed on NC (50 g l-1 sorbitol, 50 g l-1 yeast) or a regular diet (100 g l-1 sucrose, 100 g l-1 yeast) which improves fly fecundity. Locomotive activities of flies were determined by loading them into a vial (25 mm X 95 mm) capped with foam, and flies that climbed to the top of vials within 20 seconds were calculated. Procedures for lifespan assay was performed according to the literature<sup>68</sup>. Lifespan of flies was measured using 30 flies per vial each test, in which dead flies were recorded and live flies were transferred to new vials every other day.

### Fly biochemistry

For TG assay, flies were homogenized in 300 µl of 0.05% PBS-Triton, and the supernatant was heated for 10 min at 70°C to inactivate endogenous enzymes. 300 µl of Infinity TG Reagent (Thermo Electron Corp) was added to 20 µl homogenate and incubated at 37°C for 15 min. TG levels were measured as the OD value at 520 nm in Spectra Max 340 Microplate Reader and total amounts were determined using TG standard. For trehalose assay, flies

were homogenized in a buffer containing 5 mM Tris pH 6.6, 137 mM NaCl and 2.7 mM KCl, followed by a heat treatment for 10 min at 70 °C to inactivate endogenous trehalase. Half of the supernatant was diluted with an equal volume of the buffer to provide the basal value, the other half was diluted with trehalase solution (Sigma) to break trehalose into free glucose at 37 °C for 20 hours, and glucose levels were measured using Glucose (HK) Assay Kit (sigma) via reading of OD value at 340 nm. To measure the circulating trehalose, hemolymph was extracted by decapitation and centrifugation from 30–50 adult flies, and an aliquot of collected hemolymph was used to measure trehalose using the assay described above.

### Fly in situ hybridization and immunostaining

Fluorescent *in situ* hybridization was performed using a method adapted from the literature<sup>69</sup>. Briefly, 500-bp DNA fragment of *rutabaga* coding sequence was amplified from the *wild-type* drosophila cDNA by PCR and was subcloned into **pBluescript II SK** (Stratagene) vector. Digoxigenin (DIG) RNA labeling mix (Roche) was used to generate DIG-labeled antisense RNA probe. Dissected brains were fixed with 4% para-formaldehyde for 20 min and were digested with proteinase K for penetration. After blocking, the brains were hybridized with a DIG-labeled probe at 65°C overnight, incubated with anti-DIG antibody (Vector) and followed by a reaction with Alexa Fluor®565 secondary antibody (Invitrogen). An RNA probe with sense probe sequence was used as the negative control. For the immunostaining, primary rat-anti-Dilp2 was used (gift from Dr. Pierre Leopold at University of Nice, France) overnight at 4°C followed by Alexa488 anti rat-secondary antibodies (Invitrogen). DAPI staining was used to reveal all cells in the brains. Images were taken using a con-focal microscope.

### Mouse physiology and biochemistry

8–10 week-old male *C57BL/6J* and *A/J* mice purchased from the Jackson Laboratory were individually housed in a standard facility with a 12-hour light cycle and 12-hour dark cycle alternation. Mice were maintained on a normal chow before learning and memory experiments. High-fat diet (Cat #: D12451, 45 % fat, 4.73 kcal/g) and components/color-matched normal diet (Cat # D12450H, 10% fat, 3.85 kcal/g) from Research Diets were used in learning and memory test. Body weight of mice was measured every other day, and food intake was measured on a daily basis. GTT: mice were fasted overnight and then intraperitoneally injected with glucose (2 g/kg of body weight), and at time points 15, 30, 60, and 120 minutes post injection, tail vein blood glucose levels were measured with a LifeScan blood glucose monitoring system. Serum free fatty acid concentrations were measured using an enzymatic assay kit (Wako) according to the instruction in the manual. Mouse learning and memory of ND vs. HFD was tested using a procedure adapted from the literature<sup>24,49–52</sup>. Briefly, each mouse was fasted for 12-hour nighttime, and was kept for 12-hour daytime alternately in a transparent cage labeled with a blue circle sign vs. a green plus sign that was paired with high-fat diet or normal diet. One training cycle lasted 2 days, and after the desired numbers of training cycles, preference test was performed for each mouse using a test cage containing three equal compartments divided by two walls, which were the central compartment without a sign, and two side compartments that were labeled with a blue circle sign vs. a green plus sign according to the same design of the training

cages. Each mouse was placed in the center compartment for 5 min, and was then allowed to have free access to the compartments on both sides. A digital camera was used to record mouse activity and the preference to the side compartments for 15 min. The Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine approved all the procedures.

### Quantitative PCR

Total RNA was isolated using Trizol reagent (Invitrogen), and mRNA was transcribed to cDNA by using primers listed in supplementary Table 1 and M-MLV RT System (Promega). Gene expressions were analyzed using SYBR Green PCR Master Mix (Applied Biosystem). Gene expression of *Drosophila* and mouse samples were normalized to expression levels of *RP49* and  $\beta$ -*actin*, respectively.

### Statistical analysis

Two-tailed Student's t test was used for comparisons between two groups, ANOVA and appropriate post hoc analyses were used for comparisons among more than two groups, and statistical tests for each figure were justified appropriate. Sample sizes were chosen with adequate power based on the literature as well as our previous studies. Fly lifespan assay was performed using Kaplan–Meier survival analysis according to the literature<sup>68</sup>, and P values were determined by log-rank test. Maximum lifespan analysis was conducted on the longest-lived 10% of flies in each group. Data were presented as mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

We sincerely thank Dr. S. Waddell for *rut*<sup>2080</sup>, *dunce*<sup>1</sup>, and *teq*<sup>f01792</sup> mutant strains; Dr. S. Govind for UAS-*cact*; Dr. P. Leopold for anti-Dilp2 antibody; Bloomington Drosophila Stock Center for providing fly strains used in this study. This work was supported by Albert Einstein College of Medicine internal funding and NIH R01 grants AG031774, DK078750, DK099136, and HL113180 (all to D.C.).

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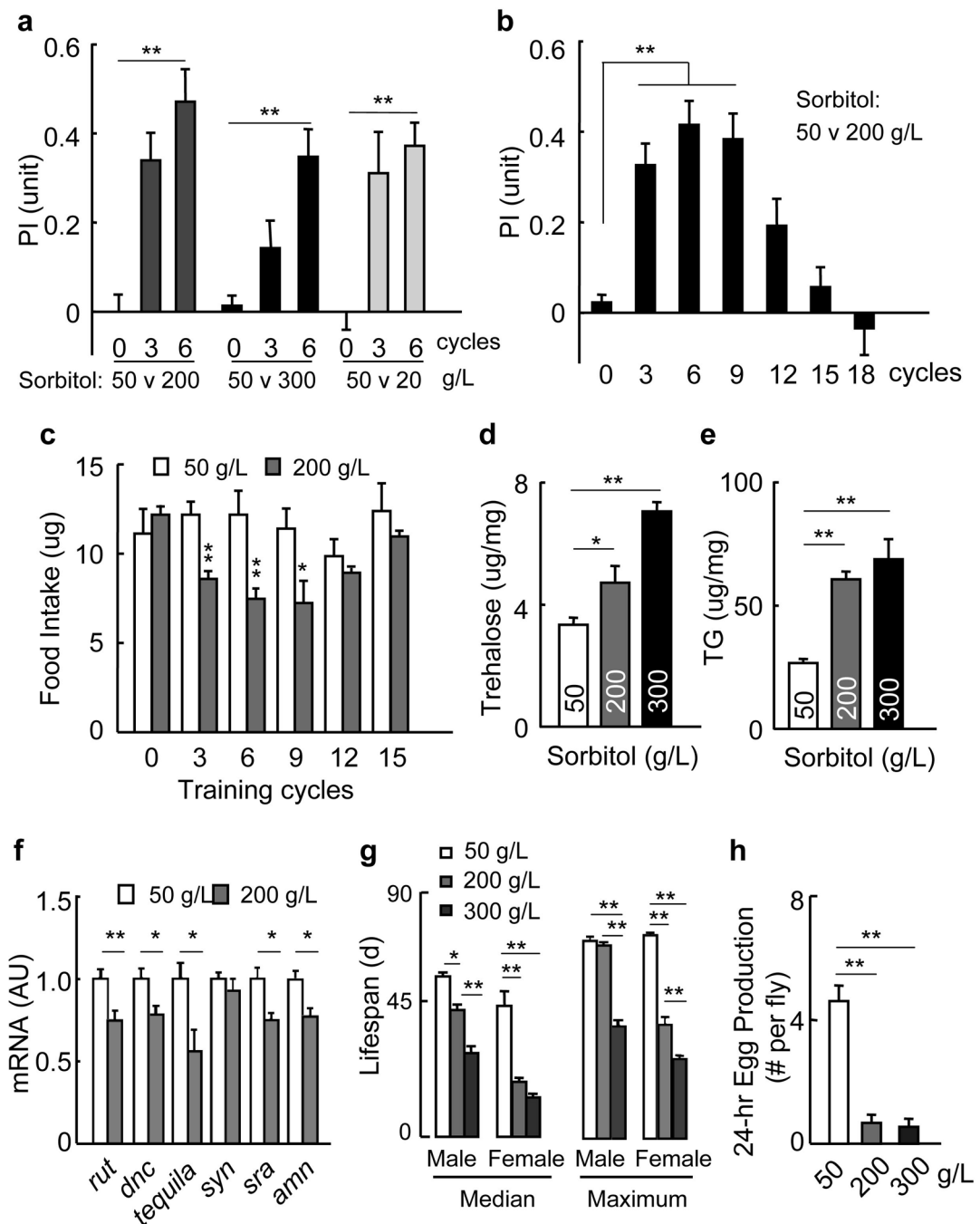
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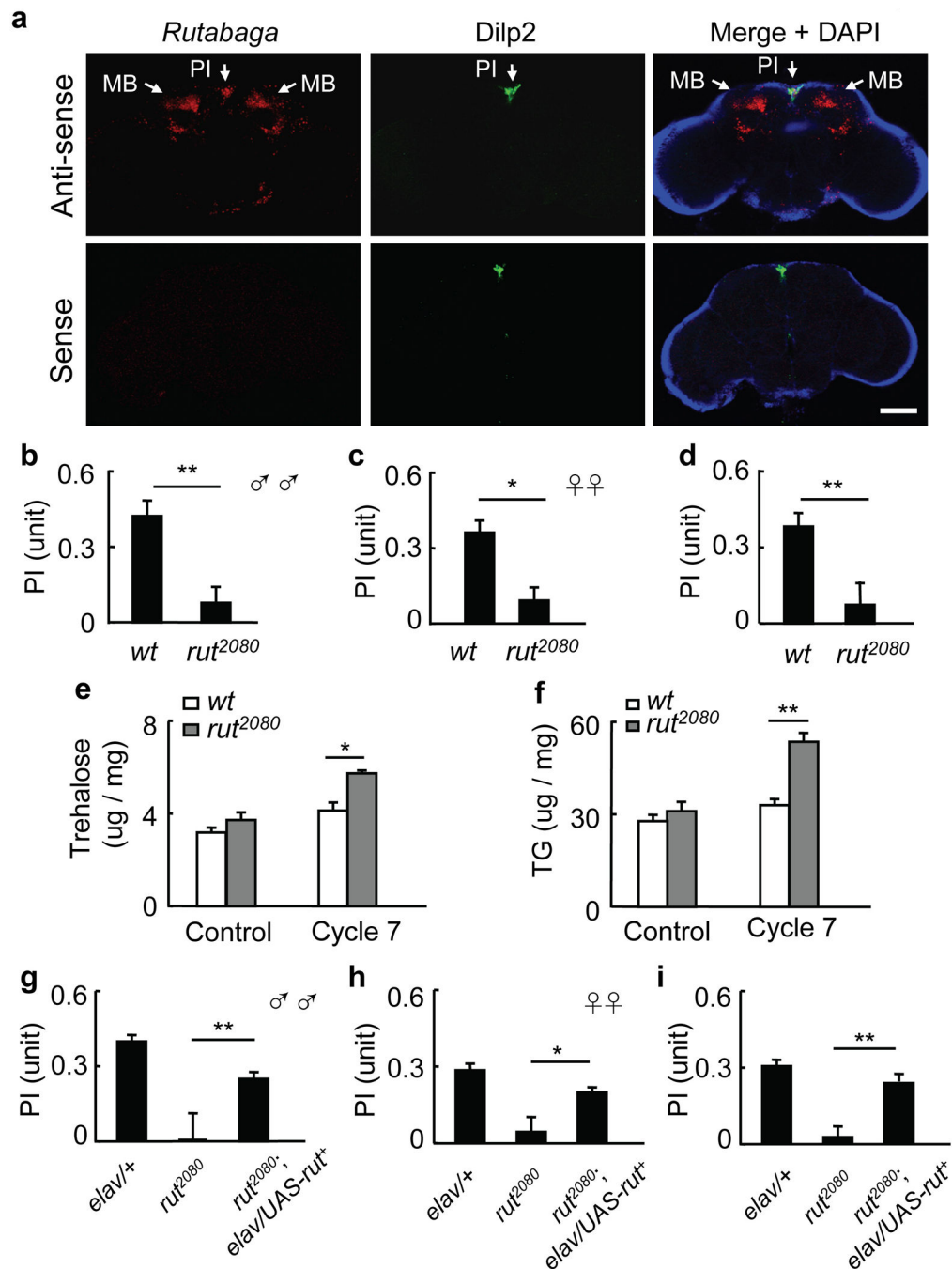
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**Figure 1. Food and metabolic control via metabolic learning and memory formation**

(a) Adult CS flies received the indicated training cycles in associating conditioned odor stimuli with normal-calorie (50 g/L sorbitol) food vs. a high-calorie (200 or 300 g/L sorbitol) or a low-calorie (20 g/L sorbitol) food, and were tested for measuring the preference index (PI).  $n = 60\text{--}80$  animals per sample, and  $n = 4\text{--}8$  samples per group. (b&c) Adult CS flies received the indicated training cycles in associating conditioned odor stimuli with normal-calorie (50 g/L sorbitol) vs. high-calorie (200 g/L sorbitol) food, and were subjected to the test for the PI (b) or measured for 30-min food intake (c).  $n = 60\text{--}80$  (b) or

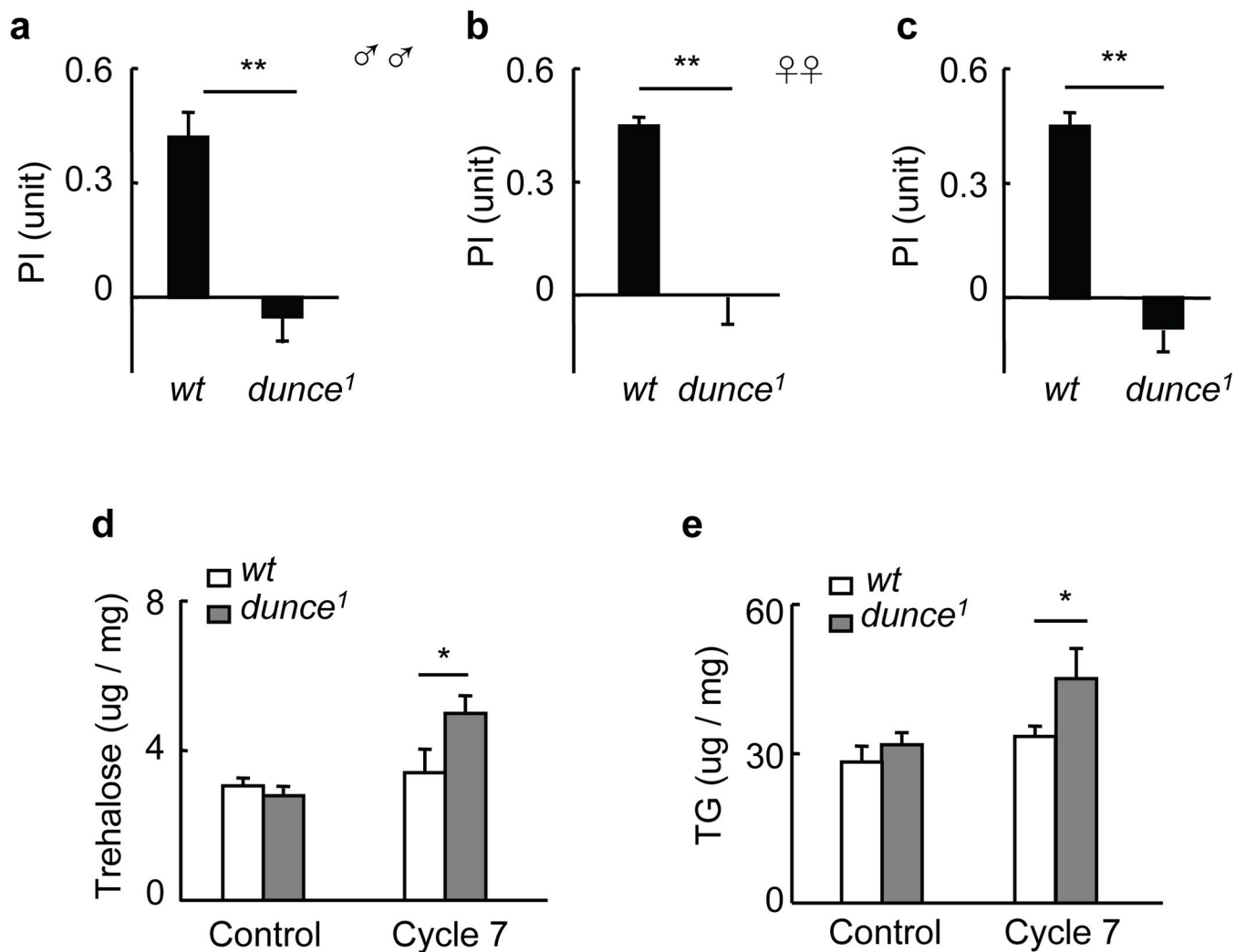
10 (e) animals per sample, and n = 4–8 samples per group. **(d&e)** Adult *CS* flies were maintained on normal-calorie (50 g/L sorbitol) vs. high-calorie (200 or 300 g/L sorbitol) food for 3 weeks. These flies were determined for body's contents of trehalose (**d**) and TG (**e**). n = 5 animals per sample, and n = 4–6 samples per group. **(f)** Adult *CS* flies were treated with normal-calorie (50 g/L sorbitol) vs. high-calorie (200 g/L sorbitol) food for 3 weeks. The mRNA levels of indicated learning/memory genes were determined. AU: arbitrary unit. n = 15 animals per sample, and n = 5–6 samples per group. **(g&h)** Adult *CS* flies were maintained on normal-calorie (50 g/L sorbitol) vs. high-calorie (200 or 300 g/L sorbitol) food. These flies were determined for lifespan (**g**) and fecundity (**h**). **g**: lifespan assay was performed using Kaplan–Meier survival analysis, and P values were determined by log-rank test. Maximum lifespan analysis was conducted on the longest-lived 10% of flies in each group. n = 30 (**g**) and 5 (**h**) animals per sample, and n = 3 (**g**) and 12 (**h**) samples per group. \*\**P* < 0.01, \**P* < 0.05 (**a, b, d–h**: ANOVA and Tukey; **c&f**: unpaired Student's t-test); error bars reflect means ± s.e.m.



**Figure 2. Requirement of *rut* gene in metabolic learning and memory formation**

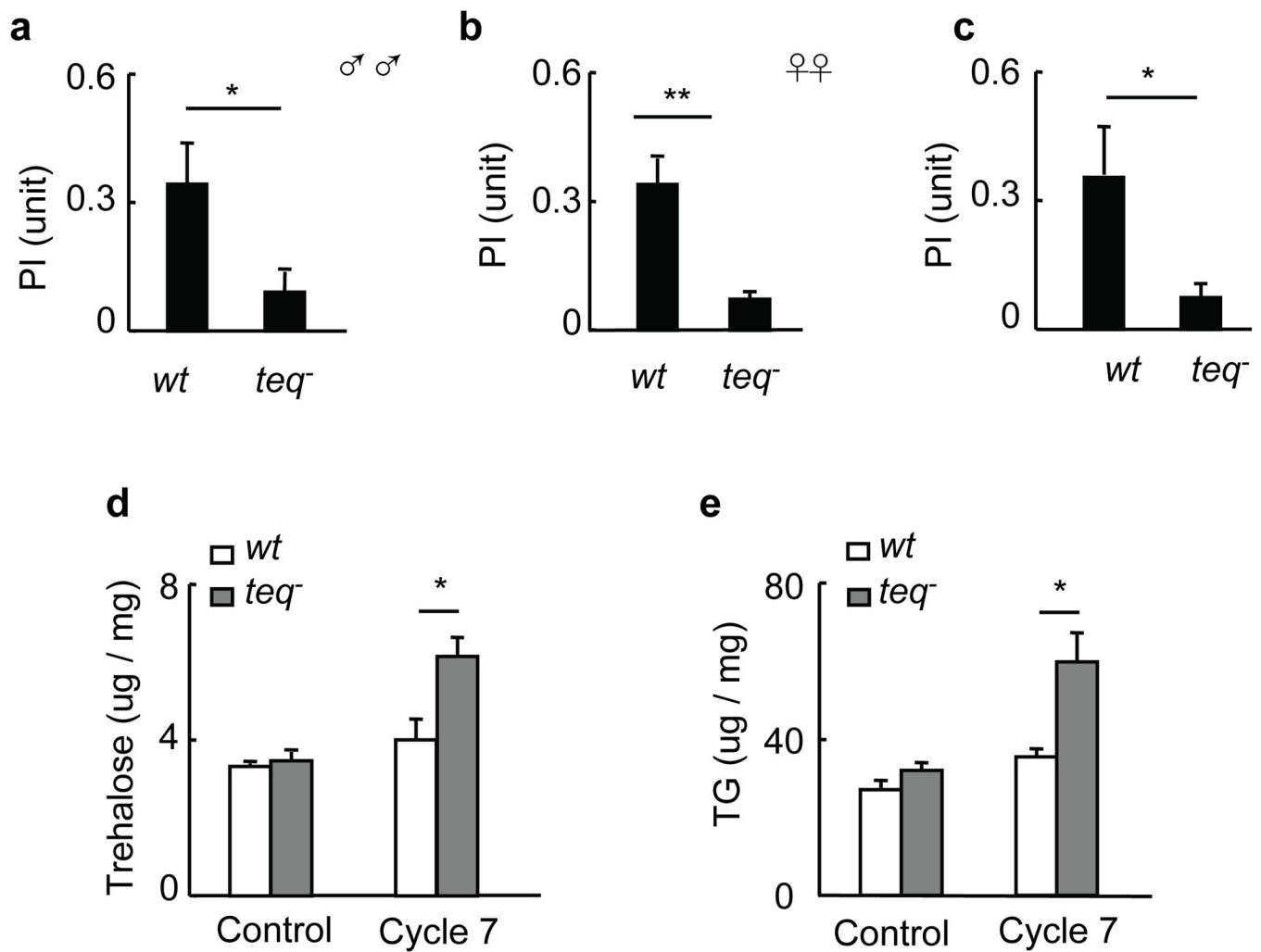
(a) Adult brains of CS flies were subjected to *in situ* hybridization for *rutabaga* mRNA (red) and immunostaining for *dilp2* antibody (green). Arrows point to the mushroom body (MB) and the pars intercerebralis (PI) in the brain. Green fluorescence indicated the PI and was merged with red staining to show *rutabaga* mRNA in this region. DAPI staining (blue) revealed the brain in this staining. Control group was stained with the sense mRNA probe and showed the absence of red staining. Bar = 100  $\mu$ m. (b–f) *rut*<sup>2080</sup> flies and wild-type (*wt*) controls received 7 cycles of training in olfactory conditioning test, and analyzed for

preference index (PI) (**b–d**) according to males (**b**), females (**c**) and both (**d**), and measured for body's contents of trehalose (**e**) and TG (**f**). Basal controls were included based on the matched flies that were fed on the same normal-calorie diet for the same training duration (**e&f**).  $n = 60\text{--}80$  (**b–d**) or 5 (**e&f**) animals per sample, and  $n = 4\text{--}6$  samples per group. (**g–i**) Flies including *rut<sup>2080</sup>*, *rut<sup>2080</sup>*; *elav-GAL4/UAS-rut<sup>+</sup>* and control *elav-GAL4/+* received 7 cycles of training in olfactory conditioning test and analyzed for preference index (PI) (**g–i**) according to males (**g**), females (**h**) and both genders (**i**).  $n = 50\text{--}70$  animals per sample, and  $n = 4\text{--}6$  samples per group.  $**P < 0.01$ ,  $*P < 0.05$  (**b–d**: unpaired Student's t-test; **e–i**: ANOVA and Tukey); error bars reflect means  $\pm$  s.e.m.



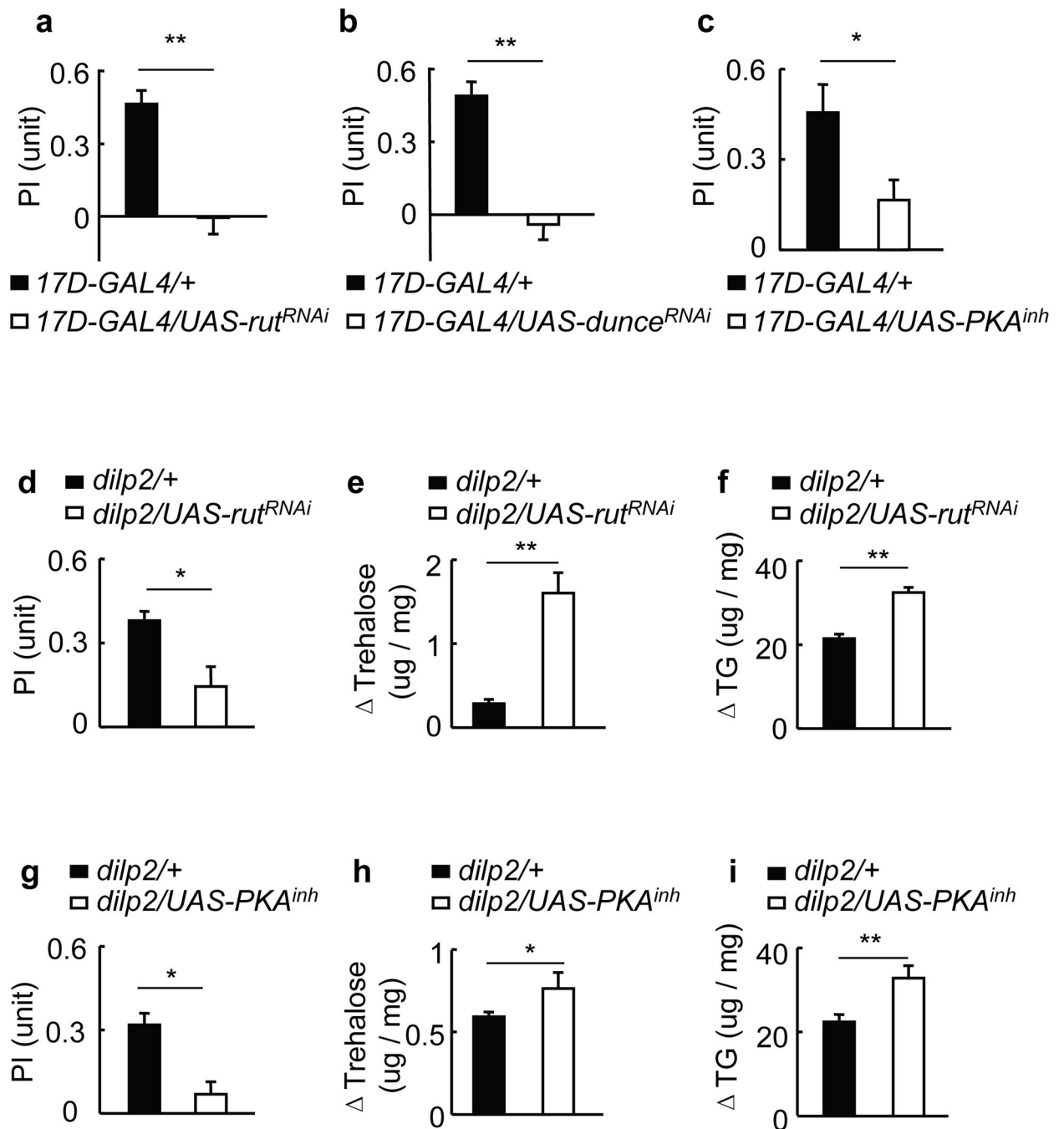
**Figure 3. Requirement of *dnc* gene in metabolic learning and memory formation**

*Dunce*<sup>1</sup> and *wt* flies received 7 cycles of training in olfactory conditioning test, and then analyzed for preference index (PI) (a–c) according to males (a), females (b) and both (c), and measured for body's contents of trehalose (d) and TG (e). Basal controls were included based on separate groups of these flies that were fed on the same normal-calorie diet for the same training duration (d, e). n = 50–70 (a–c) and 4–6 (d&e) animals per sample, and n = 4–6 samples per group. \*\**P* < 0.01, \**P* < 0.05 (a–c, unpaired Student's t-test; d&e: ANOVA and Tukey); error bars reflect means ± s.e.m.



**Figure 4. Requirement of *tequila* gene in metabolic learning and memory formation**

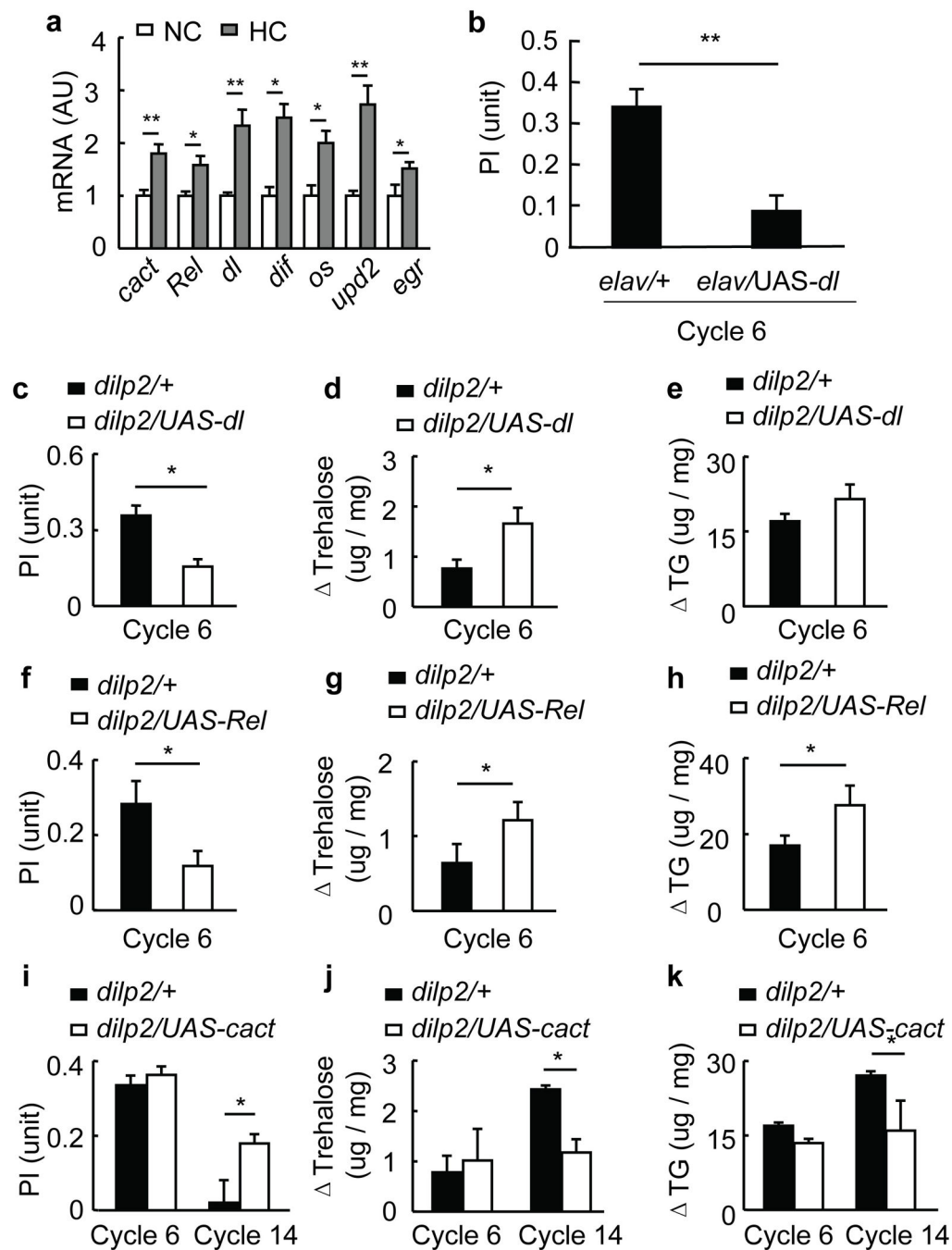
*Teq*<sup>-</sup> and *wt* flies received 7 cycles of training in olfactory conditioning test, and analyzed for preference index (PI) (a–c) according to males (a), females (b) and both (c), and measured for body's contents of trehalose (d) and TG (e). Basal controls were included based on separate groups of these flies that were fed on the same normal-calorie diet for the same training duration (d, e). n = 50–70 (a–c) and 4–6 (d&e) animals per sample, and n = 4–6 samples per group. \*\**P* < 0.01, \**P* < 0.05 (a–c: unpaired Student's t-test; d&e: ANOVA and Tukey); error bars reflect means ± s.e.m.



**Figure 5. The MB and *dilp2*-defined PI neurons in metabolic learning and memory formation** (a–c) Mutant flies including *17D-GAL4/UAS-rut*<sup>RNAi</sup> (a), *17D-GAL4/UAS-dunce*<sup>RNAi</sup> (b), *17D-GAL4/UAS-PKA*<sup>inh</sup> (c) and matched controls *17D-GAL4/+* received 6 cycles of training, and then analyzed for preference index (PI). n = 50–70 animals per sample, and n = 4–6 samples per group. (d–i) Flies including *dilp2-GAL4/UAS-rut*<sup>RNAi</sup> (shown as *dilp2/UAS-rut*<sup>RNAi</sup>) vs. matched control group *dilp2-GAL4/+* (shown as *dilp2/+*) and *dilp2-GAL4/UAS-PKA*<sup>inh</sup> (shown as *dilp2/UAS-PKA*<sup>inh</sup>) vs. matched control group *dilp2-GAL4/+* received 6–9 cycles of training, and then analyzed for preference index (PI) (d&g) and

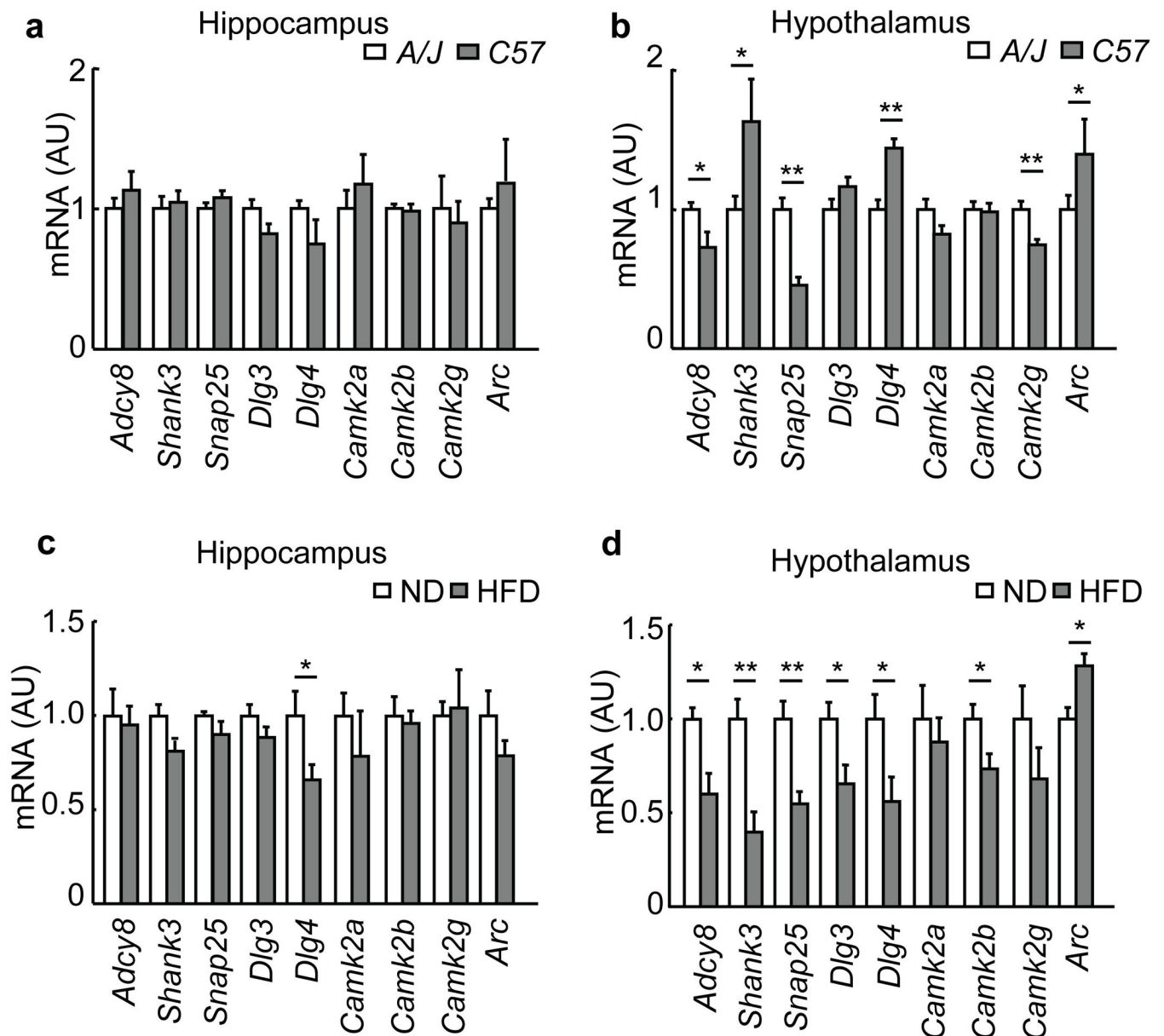


body's contents of trehalose (**e&h**) and TG (**f&i**). Data in (**e, f, h, i**) showed the change levels ( ) after training cycles compared to the levels of basal controls in which flies were maintained on the same normal-calorie diet for the same training duration. n = 40–60 (**d&g**) or 5 (**e, f, h, i**) animals per sample, and n = 4–6 samples per group. \*\* $P < 0.01$ , \* $P < 0.05$  (unpaired Student's t-test); error bars reflect means  $\pm$  s.e.m.

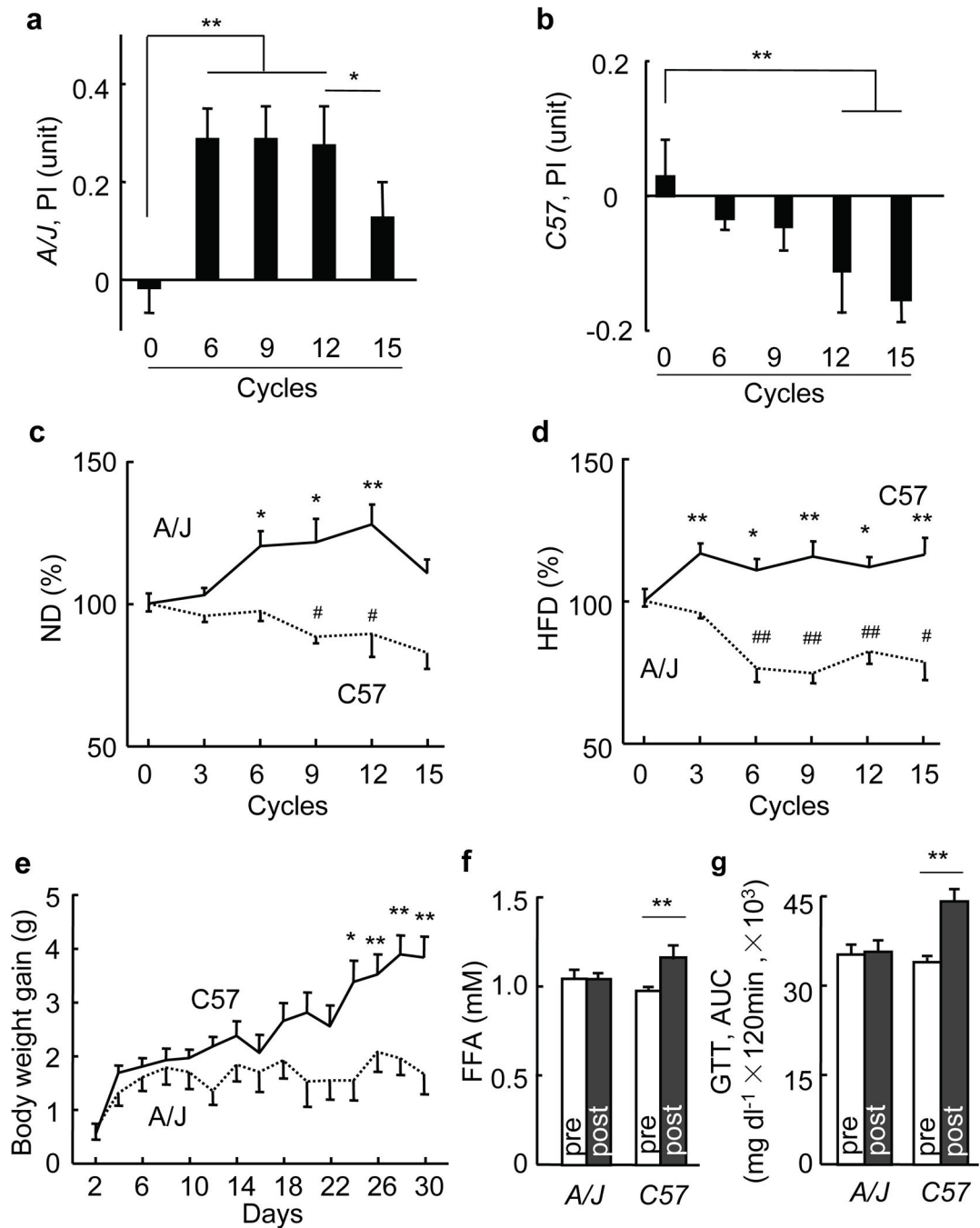


**Figure 6. Impairment of metabolic learning and memory formation by NF- $\kappa$ B pathway**  
**(a)** Brain expression levels of inflammation related genes in the adult CS flies after 18 days normal-calorie (NC, 50g/L) or high-calorie (HC, 200g/L) food treatment. AU: arbitrary unit.  $n = 30$  animals per sample, and  $n = 4$  samples per group. **(b–k)** Flies *elav-GAL4/UAS-dl* (shown as *elav/UAS-dl*) vs. control *elav-GAL4/+* (shown as *elav/+*) **(b)**, *dilp2-GAL4/UAS-dl* (shown as *dilp2/UAS-dl*) vs. control *dilp2-GAL4/+* **(c–e)**, *dilp2-GAL4/UAS-Rel* (shown as *dilp2/UAS-Rel*) vs. control *dilp2-GAL4/+* **(f–h)**, and *dilp2-GAL4/UAS-cact* (shown as *dilp2/UAS-cact*) vs. control *dilp2-GAL4/+* **(i–k)** received indicated cycles of training, and then

analyzed for preference index (PI) (**b, c, f, i**) and body's contents of trehalose (**d, g, j**) and TG (**e, h, k**). Data in (**d, e, g, h, j, k**) showed the change levels ( ) after indicated training cycles compared to the levels of basal controls in which flies were maintained on the same normal-calorie diet for the same training duration.  $n = 50-70$  (**b, c, f, i**) or 5 (**d, e, g, h, j, k**) animals per sample, and  $n = 4-8$  samples per group.  $**P < 0.01$ ,  $*P < 0.05$  (**a-h**, unpaired Student's t-test; **i-k**: ANOVA and Tukey); error bars reflect means  $\pm$  s.e.m.

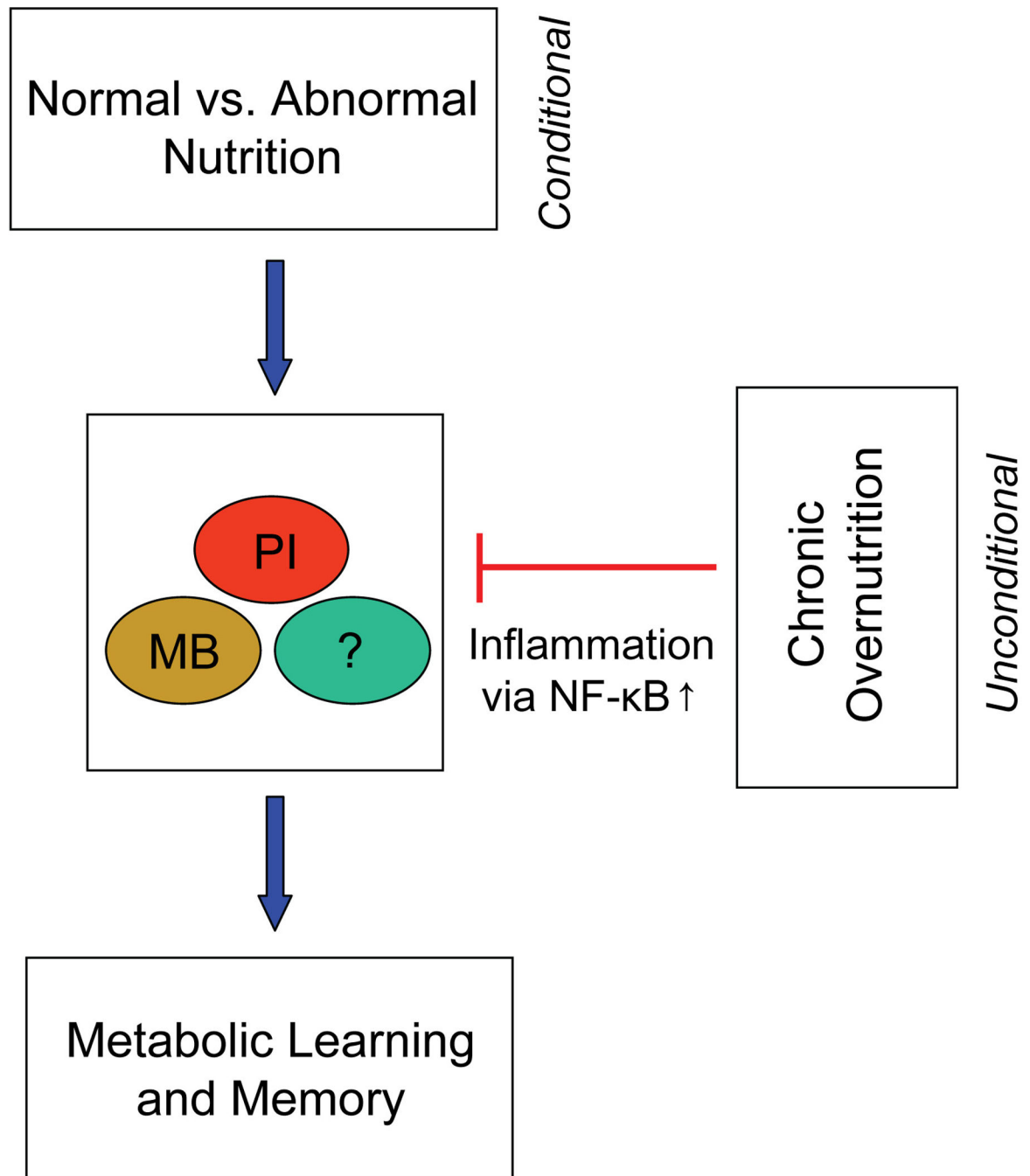


**Figure 7. Involvement of the hypothalamus in learning/memory-regulating genes of mice** (a&b) Hippocampal (a) and hypothalamic (b) levels of learning/memory genes in *A/J* and *C57BL/6J* mice (~ 3-month-old males) under basal and normal chow feeding conditions. (c&d) The mRNA levels of learning/memory genes were determined for the hypothalamus (b) and hippocampus (c) of adult male *C57BL/6J* mice that received 3-month normal diet (ND) vs. high-fat diet (HFD) feeding. \*\* $P < 0.01$ , \* $P < 0.05$  (unpaired Student's t-test),  $n = 4-6$  mice per group; error bars reflect means  $\pm$  s.e.m.



**Figure 8. Different metabolic learning/memory profiles in A/J vs. C57BL/6J mice**  
**(a&b)** Preference Index (PI) of A/J **(a)** and C57BL/6J **(b)** mice following indicated training cycles. The PI was calculated as the time difference that mice spent in a cage of a conditioned stimulus associated with normal diet (ND) vs. high-fat diet (HFD) divided by the total time. **(c&d)** Percentage changes of ND intake **(c)** vs. HFD intake **(d)** in A/J and C57BL/6J mice at the indicated training cycles relative to food intake at training cycle 0. Statistics reflect comparison with 0 cycle in each strain. **(e)** Body weight gain of A/J and C57BL/6J mice at the indicated days during the training. Statistics reflect comparisons

between C57 and A/J at matched time points. **(f)** Serum FFA in *C57BL/6J* and *A/J* prior to training (Pre) vs. post 21 training cycles (Post). **(g)** Calculated values for area under curve (AUC) during 120-min glucose tolerance test (GTT) of *A/J* and *C57BL/6J* mice prior to training (Pre) vs. post 21 training cycles (Post).  $n = 8-10$  (**a-e**) and  $n = 5-8$  mice (**f, g**) per group.  $**P < 0.01$ ,  $##P < 0.01$ ,  $*P < 0.05$ ,  $\#P < 0.05$  (ANOVA and Tukey); error bars reflect means  $\pm$  s.e.m.



**Figure 9. Working model of metabolic learning and memory formation**

Under an environmental condition containing normal and abnormal nutrition, *Drosophila* can develop a form of learning and memory of metabolic information, referred to as “metabolic learning & memory”, to mediate the central control of metabolic homeostasis, which requires cooperative actions of the MB and PI neurons. However, chronic overnutrition can employ NF-κB-driven neuroinflammation to impair this form of learning

and memory, contributing to the development of overnutrition-induced metabolic disorders and diseases.

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